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14. ABSTRACT

Intraductal "foam cells" are the most commonly encountered cells in spontaneous nipple discharge, aspirates, and lavage, and frequently surround DCIS and other intraductal proliferations. The origin of these cells is thus of potential importance, but is not presently understood. This project tested the hypothesis that these intraductal macrophages take origin from bone marrow-derived hematopoietic precursors. The central work tests the idea by transfer of bone marrow from C57BL/6 mice recombinantly expressing green fluorescent protein (GFP) into wildtype GFP- recipients. After transfer, mice were hormonally manipulated for mammary epithelial stimulation, and evaluated for recruitment of GFP+ cells (hematopoeitic origin) associated with mammary epithelium. The prediction of foam cells with hematopoeitic origin was not confirmed. However, we showed that precursor cancer stem cells of hematopoeitic origin developed in mammary tissue with both benign and malignant differentiation, depending on environmental cues. Progression of the cells to cancer is associated with the up-regulation of c-kit and Sca-1, and was regulated by the PIWI/AGO family gene piwil2. This demonstrates a surprising contribution of hematopoeitic precursors to the heterogeneity of cell types in benign and malignant mammary tissue.

15. SUBJECT TERMS
Mammary tissue, foam cells, green fluorescent protein, cancer stem cells

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Introduction

Intraductal "foam cells" are the most commonly encountered cells in spontaneous nipple discharge, nipple aspirate fluid and ductal lavage yet their origin and significance remain a mystery. These cells increase in human and murine pregnancy and other conditions of ductal ectasia and /or obstruction. They frequently surround DCIS and other intraductal proliferations but their presence has been regarded, more or less, as a nuisance since they often hide the diagnostically more important epithelial cells. Our previous immunocytochemical studies with macrophage (CD68, lysozyme), epithelial (cytokeratin., estrogen receptor) and myoepithelial (smooth muscle actin, CALLA, maspin) markers have indicated that foam cells are of macrophage lineage and terminally differentiated (negative Ki-67 and PCNA). Studies of others have confirmed these observations (3). We have also observed in humans other variants of these intraductal macrophages which exhibit not foam vacuoles of milk/milk products but phagocytosed endogenous intraductal debris including erythrocytes/hemosiderin and exogenous materials including barium and gatrogaffin introduced by ductal lavage.

Because these macrophages are observed only intraductally and because their appearance resembles lactating and vacuolated epithelial cells, their origin has been presumed to be of ductal lining epithelium. However this has not been proven. The origin and significance of mammary intraductal foam cells remain an important and unanswered question warranting study. Accordingly, this project was designed to test the hypothesis that these intraductal macrophages take origin from bone marrow-derived hematopoietic precursors. Our work involved the murine bone marrow transplant model, due to the robustness of experimental reagents and genetically-modified mouse strains suitable for resolving this developmental question.

Body

In our initial studies, bone marrow from wild type male C57 black mice and green fluorescent protein (GFP) transgenic male C57 black mice (Jackson Laboratories) were harvested by femoral flushing. In the former group, the bone marrow were labeled ex vivo by retroviral transfection of GFP using retroviral vector optimized for gene expression in hematopoietic stem cells. In both groups, the harvested marrow was tail-vein injected into lethally irradiated female C57 mice (to ablate recipient marrow). Mice exhibiting successful bone marrow engraftment of at least 50% donor marrow were identified, and made pseudopregnant with injections of progesterone. Mammary fat pads were excised and examined for the presence of GFP-positive intraductal macrophages. In this line of experimentation, we were unable to document the presence of intraductal macrophages derived from hematopoeitic origin by this criterion. While these results do not rule out the primary prediction of this study, we have not derived data to this point that supports the hypothesis.

We therefore chose to address more broadly the idea that cells of hematopoeitic origin, notably cancer stem cells (CSCs), may contribute to physiologic cell types resident in the mammary and other tissues. We took advantage of mice with the targeted mutation of p53 and stat-1 genes, whose hematopoeitic cells progress to dendritic cell (DC)-like leukemia. After cloning DC-like lines from the spleen of leukemic mice, we isolated clones that expressed neither hematopoietic and lineage (Lin) markers nor hematopoietic stem cell (HSC) markers (CD45−c-kit−Sca-1−Lin−). In short, these cells had the potential for both benign and malignant differentiation, and their fate was determined by environmental cues. These findings were recently reported (Chen L et al., Precancerous stem cells have the potential for both benign and malignant differentiation. 2007. PLoS ONE. 14;2:e293.

<u>pCSCs exhibit stem-like cell phenotype</u>
Although cancer stem-like cells can be detected in the existing tumor cell lines, no clonal CSC lines have been established. In this study, we found that 3 of the 25 clones (2C4, 3B5C and 3B6C) failed to express hematopoietic pan-marker CD45 and lineage (Lin) markers CD3ε, CD4, CD8, B220, Ter-119, CD11b and Gr-1. Cytological analysis demonstrated that all the clones exhibited blast-like cell morphology with large numbers of cytoplasmic vacuoles or granules. Overall, these clones exhibit a stem-like cell phenotype CD45−c-kit−Sca-1−Lin−CD44high (CD45KSL−CD44high). Cytogenetic analysis revealed that all the three clones carried an identical pseudodiploid karyotype with multiple chromosomal translocations.

To test whether the pCSCs have the activity of hematopoietic progenitors, we evaluated the multipotency of pCSCs using the colony-forming cell (CFC) assay. About 30~50% of the input cells had colony-forming activity (CFU) in the medium of Methocult GF M3434. Although we did not observe all types of CFUs that may be differentiated from normal HSCs, such as burst forming units-erythroid (BFU-E), CFU-M (macrophage), and CFU-G (granulocyte), three types of CFUs were identified from these clones, including CFU-E (erythroid), CFU-mix, and CFU-GM. These results suggest that the normal development program of the pCSCs was dramatically impaired, but not completely abolished.

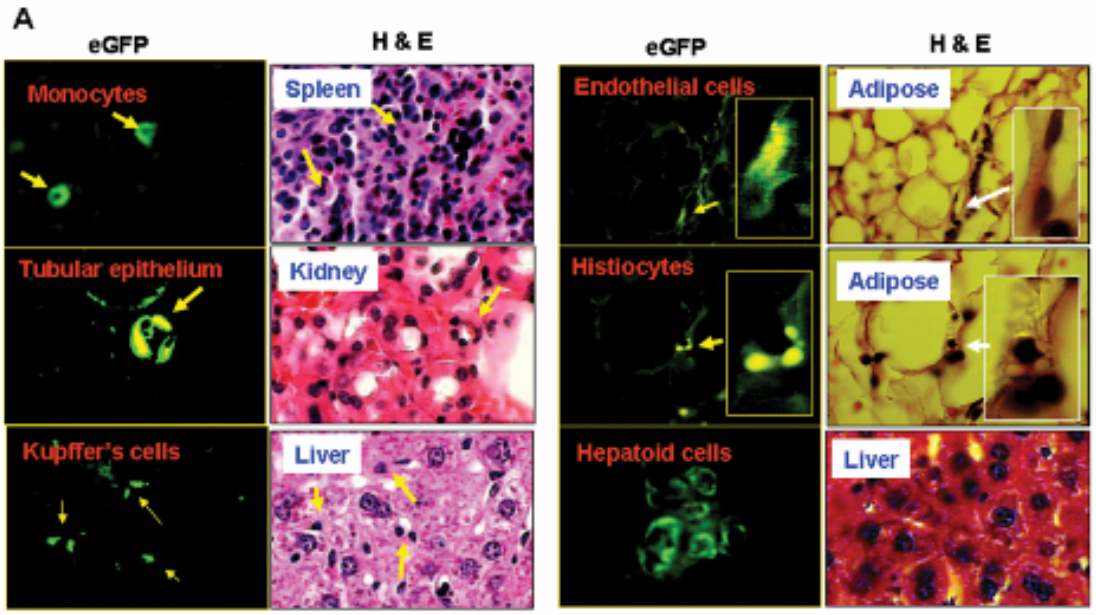
To evaluate long-term repopulating activity, lethally irradiated CD45.1+ congenic B6 mice were injected i.v. with both 2C4, 3B5C or 3B6C cells and recipient-type bone marrow (BM) cells. Donor-derived CD45.2+ lymphoid (CD3ε+) and myeloid (CD11b+ or Gr-1+) cells in the peripheral blood were monitored by flow cytometry beginning ~4 wks post-transplant. CD45.2+ donor cells were not significantly detected until ~8 wks after transfer. About 0.5~10% more CD45.2+CD11b+ and CD45.2+Gr-1+ cells were detected, depending on individual mice or experiments (5~10 mice/expt). The incomplete differentiation may explain the low myeloid engraftment, as well as the absence of lymphoid engraftment.

pCSCs can differentiate into various types of nonmalignant cells, or of malignant phenotype

The lethally irradiated mice receiving both pCSCs and BM cells survived tumor-free for up to 10 months. This indicated that the pCSCs either distributed in various organs in a quiescent status or differentiated into tissue-specific cells in these organs or tissues. To test these two possibilities, we stably transduced 2C4 cells with lentiviruses, which carried an enhanced green fluorescent protein (GFP) gene, to track the fate of pCSCs in recipients. The donor-derived GFP+ cells, albeit lower in frequency, were readily detected in various organs, such as the spleen, liver, kidney, small intestine, or adipose tissues, of all the mice that had received 2C4G2, but not 2C4 cells, for 5 months (Fig. 1a and data not shown). Some GFP+ cells exhibited the morphology of tissue origin, including endothelial cells, tubular epithelial cells, Kupffer's cells, histiocytes, macrophages/monocytes, and hepatoid cells (Fig. 1a). In the liver, eGFP+ Kupffer's cells and hepatoid cells were usually found in the regenerative areas. Interestingly, none of the observed eGFP+ cells exhibited significant dysplastic changes. The results suggest that some pCSCs could differentiate into tissue-specific cells in a regenerative environment. Similar findings were observed with blastocyst chimeric mice (Fig. 1b-e).

Interestingly, none of the recipients developed tumors under these conditions in immunocompetant mice. However, when injected into immunodeficient mice, malignancies developed both as leukemias and as solid tumors of epithelial or stromal phenotype. Since the pCSCs exhibited the properties of stem cells, as well as the potency of tumorigenesis, we examined the expression of embryonic and adult stemness-related genes. The embryonic stem cell-related genes, including Pouf1/Otc4, TDGF1, Zfp42/REX1 and Mili (piwil2), whose homologue has a conserved function in stem cell division, were exclusively expressed in pCSCs. Among them, only mili was stably expressed in all the clones of pCSCs; in contrast, miwi, a member of mouse PIWI/AGO gene family, was not detectable in these pCSCs. The unique pattern of mili and miwi mRNA in pCSCs does not seem to be associated with the deficiency of p53 and Stat-1, because we could not detect either gene in the p53-null embryonic fibroblasts or the Stat-1-null hematopoietic cells. The results suggest that mili may play an important role in pCSC development.

Figure 1. A, Differentiation of pCSCs into hematopoietic and non-hematopoietic cells: The lethally irradiated CD45.1 congenic B6 mice were injected i.v. with 1×106 2C4 or GFP+ 2C4G2 cells, along with 5×105 recipient-type BM cells. Mice were sacrificed 5 months post transfer. Organs were harvested, fixed in 10% formaldehyde of PBS, prepared for H & E. staining, and examined under fluorescent microscope. The morphology of GFP+ cells was determined under bright field. B–E, Development of pCSCs in blastocyst chimeric mice: E3.5 dpc of FVB mice were injected with 2C4G2 (8~10 cells per blastocyst) and transferred to pseudopregnant surrogate mothers. The progeny were delivered and grew to adult without any complication. The data shown are from one of two experiments in which 8 progeny (male: n = 6; female: n = 2) were obtained. One male mouse died of fighting at 3 months of age. B, GFP+ RBCs in 7/8 of the chimeric mice: The data represent the air-dried blood smears from two mice, at the age of 2 months, examined under, respectively, the bright and fluorescent fields of a fluorescent microscope (Nike, E400, Japan). C, pCSC-derived eGFP+CD45+ cells: peripheral blood was harvested from the chimeric mice at age 2 months (n = 6; other two pregnant mice were not examined) or control FVB mice (n = 10), stained with PE-conjugated mAb to CD45, and analyzed by flow cytometry. D & E, Living image of the chimeric mice: A representative living image of the chimeric mice at 4 months of age is shown in D, demonstrated by IVIS imaging systems incorporated with Living Imaging® software (Xenogen Inc.); and the eGFP-derived photon counts in the region of interest (ROI) of 7 mice are shown in E. Normal FVB mice were used as control for living imaging.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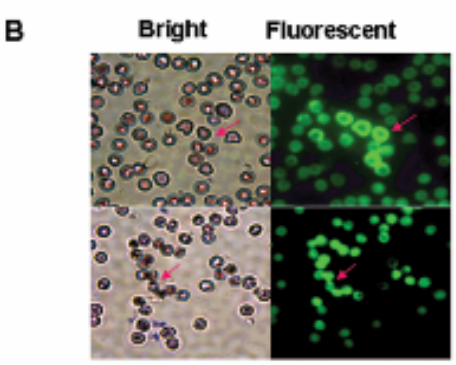
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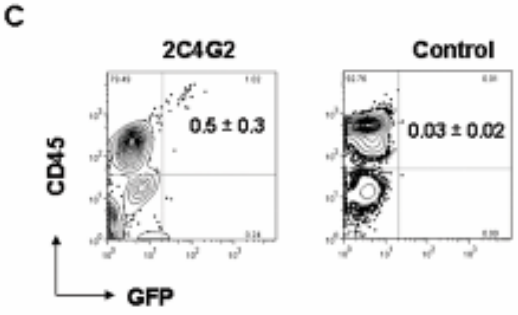
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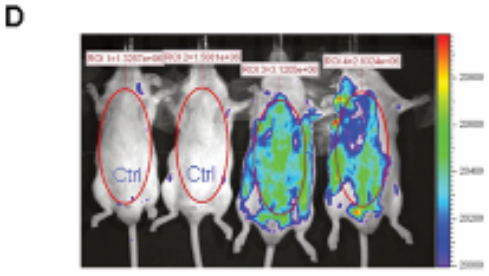
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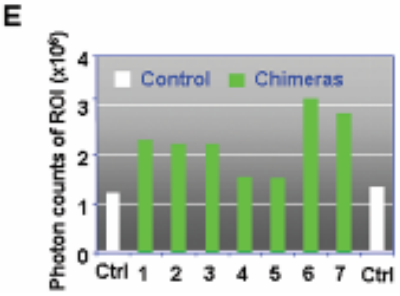
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Key research accomplishments

- Precursor cancer stem cell lines of hematopoeitic origin can be isolated with both benign and malignant differentiation
- Environmental cues, notably the presence of immunocompetant cell types, determine differentiative and malignant fate
- Progression of the cells to cancer is associated with the up-regulation of c-kit and Sca-1, regulated by the PIWI/AGO family gene piwil2.
- This demonstrates a surprising contribution of hematopoeitic precursors to the heterogeneity of cell types in benign and malignant mammary tissue.

Reportable outcomes.

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Conclusions.

This project demonstrates a surprising contribution of hematopoeitic precursors to the heterogeneity of cell types in benign and malignant mammary tissue. A broad therapeutic approach to the cure of various types of cancers may be achievable through rational targeting of pCSCs.

References. None